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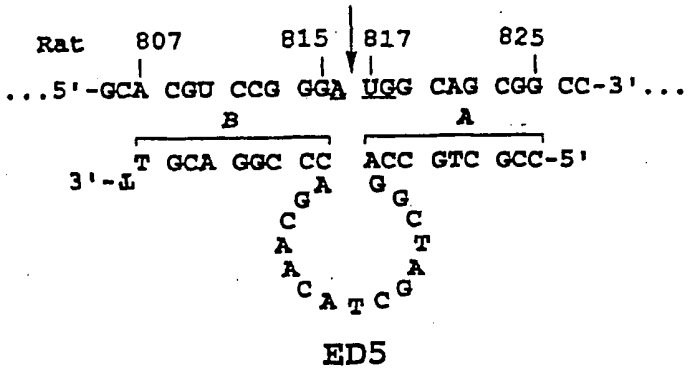
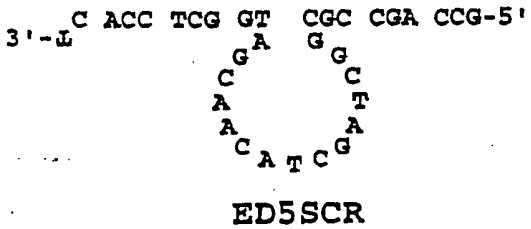
(54) Title: CATALYTIC MOLECULES

(57) Abstract

The present invention relates to DNAzymes which are targeted against mRNA molecules encoding EGR-1 (also known as Egr-1 or NGFI-A). The present invention also relates to compositions including these DNAzymes and to methods of treatment involving administration of the DNAzymes.





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CATALYTIC MOLECULES

FIELD OF THE INVENTION

The present invention relates to DNAzymes which are targeted against mRNA molecules encoding EGR-1 (also known as Egr-1 or NGFI-A). The present invention also relates to compositions including these DNAzymes and to methods of treatment involving administration of the DNAzymes.

BACKGROUND OF THE INVENTION

Egr-1 expression in Smooth Muscle Cells

Smooth muscle cells (SMCs) are well recognized as a significant cellular component of atherosclerotic and post-angioplasty restenotic lesions (Stary et al, 1995; Holmes et al, 1984). SMC migration and proliferation are key events in the pathogenesis of these vascular disorders (Jackson & Schwartz, 1992; Libby et al, 1995). The promoter regions of many genes that encode mitogenic and migratory factors expressed by SMCs in these lesions (Evanko et al, 1998; Murry et al, 1996; Ueda et al, 1996; Tanizawa et al, 1996; Rekhter & Gordon, 1994; Hughes et al, 1993; Brogi et al, 1993; Wilcox et al 1989; Wilcox et al, 1988) contain nucleotide (nt) recognition elements for the nuclear protein and transcription factor, Egr-1 (Khachigian & Collins, 1997; Khachigian et al, 1996). Egr-1 is not expressed in the unmanipulated artery wall, but is rapidly activated by mechanical injury (Khachigian et al, 1996; Silverman et al, 1997; Kim et al, 1995). It is also induced in vascular endothelial cells and/or SMCs exposed to fluid biomechanical forces (Khachigian et al, 1997; Sumpio et al, 1998) and multiple other pathophysiologically-relevant agonists (Delbridge & Khachigian, 1997).

DNAzymes

In human gene therapy, antisense nucleic acid technology has been one of the major tools of choice to inactivate genes whose expression causes disease and is thus undesirable. The anti-sense approach employs a nucleic acid molecule that is complementary to, and thereby hybridizes with, an mRNA molecule encoding an undesirable gene. Such hybridization leads to the inhibition of gene expression.

Anti-sense technology suffers from certain drawbacks. Anti-sense hybridization results in the formation of a DNA/target mRNA heteroduplex. This heteroduplex serves as a substrate for RNAse H-mediated degradation of the target mRNA component. Here, the DNA anti-sense molecule serves in a passive manner, in that it merely facilitates the required cleavage by endogenous RNAse H enzyme. This dependence on RNAse H confers limitations on the design of anti-sense molecules regarding their chemistry and ability to form stable heteroduplexes with their target mRNA's. Anti-sense DNA molecules also suffer from problems associated with non-specific activity and, at higher concentrations, even toxicity.

As an alternative to anti-sense molecules, catalytic nucleic acid molecules have shown promise as therapeutic agents for suppressing gene expression, and are widely discussed in the literature (Haseloff (1988); Breaker (1994); Koizumi (1989); Otsuka; Kashani-Sabet (1992); Raillard (1996); and Carmi (1996)). Thus, unlike a conventional anti-sense molecule, a catalytic nucleic acid molecule functions by actually cleaving its target mRNA molecule instead of merely binding to it. Catalytic nucleic acid molecules can only cleave a target nucleic acid sequence if that target sequence meets certain minimum requirements. The target sequence must be complementary to the hybridizing regions of the catalytic nucleic acid, and the target must contain a specific sequence at the site of cleavage.

Catalytic RNA molecules ("ribozymes") are well documented (Haseloff (1988); Symonds (1992); and Sun (1997)), and have been shown to be capable of cleaving both RNA (Haseloff (1988)) and DNA (Raillard (1996)) molecules. Indeed, the development of in vitro selection and evolution techniques has made it possible to obtain novel ribozymes against a known substrate, using either random variants of a known ribozyme or random-sequence RNA as a starting point (Pan (1992); Tsang (1994); and Breaker (1994)).

Ribozymes, however, are highly susceptible to enzymatic hydrolysis within the cells where they are intended to perform their function. This in turn limits their pharmaceutical applications.

Recently, a new class of catalytic molecules called "DNAzymes" was created (Breaker and Joyce (1995); Santoro (1997)). DNAzymes are single-stranded, and cleave both RNA (Breaker (1994); Santoro (1997)) and DNA (Carmi (1996)). A general model for the DNAzyme has been proposed, and is known as the "10-23" model. DNAzymes following the "10-23" model, also

referred to simply as "10-23 DNAzymes", have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. In vitro analyses show that this type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions under physiological conditions (Santoro (1997)).

DNAzymes show promise as therapeutic agents. However, DNAzyme success against a disease caused by the presence of a known mRNA molecule is not predictable. This unpredictability is due, in part, to two factors. First, certain mRNA secondary structures can impede a DNAzyme's ability to bind to and cleave its target mRNA. Second, the uptake of a DNAzyme by cells expressing the target mRNA may not be efficient enough to permit therapeutically meaningful results. For these reasons, merely knowing of a disease and its causative target mRNA sequence does not alone allow one to reasonably predict the therapeutic success of a DNAzyme against that target mRNA, absent an inventive step.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the present invention provides a DNAzyme which specifically cleaves EGR-1 mRNA, the DNAzyme including

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:1, such that the DNAzyme cleaves the EGR-1 mRNA.

In a second aspect the present invention provides a pharmaceutical composition including a DNAzyme according to the first aspect and a pharmaceutically acceptable carrier.

In a third aspect the present invention provides a method of inhibiting EGR-1 activity in cells which includes exposing the cells to a DNAzyme according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of inhibiting proliferation or migration of cells in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In a fifth aspect the present invention provides a method of treating a condition associated with cell proliferation or migration in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In a sixth aspect the present invention provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to the first aspect.

In a seventh aspect, the present invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to the fifth aspect to the subject at around the time of the angioplasty.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Sequence of NGFI-A DNAzyme (ED5), its scrambled control (ED5SCR) and 23 nt synthetic rat substrate. The translational start site is underlined.

Figure 2 NGFI-A DNAzyme inhibits the induction of NGFI-A mRNA and protein by serum. Northern blot analysis was performed with 25 μg of total RNA. The blot was stripped and reprobed for β-Actin. Autoradiograms were analyzed by scanning densitometry and the ordinate axis is expressed as NGFI-A band intensity as a fraction of β-Actin band intensity. The mean and standard errors of the mean are indicated in the figure. Data is representative of 2 independent experiments. * indicates $P<0.05$ (Student's paired t-test) as compared to control (FBS alone).

Figure 3 SMC proliferation is inhibited by NGFI-A DNAzyme. **a**, Assessment of total cell numbers by Coulter counter. Growth-arrested SMCs that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of AS2 is 5'-CTT GGC CGC TGC CAT-3' (SEQ ID NO: 20). **b**, Proportion of cells incorporating Trypan Blue after exposure to serum and/or DNAzyme. Cells were stained incubated in 0.2% (w:v) Trypan Blue at 22 °C for 5 min prior to quantitation by hemocytometer in a blind manner. **c**, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum and/or DNAzyme for 3 days were resuspended and numbers were quantitated by Coulter counter. Data is representative of 2 independent experiments performed in triplicate. The mean and standard errors of the mean are indicated in the figure. * indicates $P<0.05$ (Student's paired t-test) as compared to control (FBS alone).

Figure 4 NGFI-A DNAzyme inhibition of neointima formation in the rat carotid artery. Neointimal and medial areas of 5 consecutive sections per rat (5 rats per group) taken at 250 μm intervals from the point of ligation were determined digitally and expressed as a ratio per group. The mean and standard errors of the mean are indicated by the ordinate axis. * denotes

$P<0.05$ as compared to the Lig, Lig+Veh or Lig+Veh+ED5SCR groups using the Wilcoxen rank sum test for unpaired data. Lig denotes ligation, Veh denotes vehicle.

Figure 5 Selective inhibition of human smooth muscle cell proliferation by DzA.

Figure 6 Specific inhibition of porcine retinal smooth muscle cell proliferation by DzA.

DETAILED DESCRIPTION OF THE INVENTION

Egr-1 (also known as NGFI-A and EGR-1) binds to the promoters of genes whose products influence cell movement and replication in the artery wall. Table 1 shows an alignment of the human EGR-1 cDNA sequence with the equivalent mouse (Egr-1) and rat (NGFI-A) sequences. The present inventors have now developed DNA-based enzymes that cut NGFI-A/Egr-1/EGR-1 RNA with high efficiency and specificity. The NGFI-A "DNAzyme" cleaved synthetic and *in vitro* transcribed NGFI-A RNA in a sequence-specific manner and inhibited production of NGFI-A in vascular smooth muscle cells without influencing levels of the related zinc finger protein, Sp1, or the immediate-early gene product, c-Fos. The DNAzyme blocked serum-inducible DNA synthesis in smooth muscle cells and attenuated total cell proliferation. The DNAzyme also inhibited the reparative response to mechanical injury, both in culture and in the rat carotid artery wall. These results indicate a necessary and sufficient role for NGFI-A/Egr-1/EGR-1 in vascular smooth muscle cell growth and provide the first demonstration of a DNAzyme targeted against NGFI-A/Egr-1/EGR-1 transcripts.

Accordingly, in a first aspect the present invention provides a DNAzyme which specifically cleaves EGR-1 mRNA, the DNAzyme including

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:1, such that the DNAzyme cleaves the EGR-1 mRNA.

As used herein, "DNAzyme" means a DNA molecule that specifically recognizes and cleaves a distinct target nucleic acid sequence, which may be either DNA or RNA.

In a preferred embodiment of the first aspect of the present invention, the binding domains are complementary to the regions immediately flanking the cleavage site. It will be appreciated by those skilled in the art, however,

that strict complementarity may not be required for the DNAzyme to bind to and cleave the EGR-1 mRNA.

The catalytic domain of a DNAzyme of the present invention may be any suitable catalytic domain. Examples of suitable catalytic domains are described in *Santoro and Joyce, 1997* and US 5807718, the entire contents of which are incorporated herein by reference. In a preferred embodiment, the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO: 2).

Within the parameters of the present invention, the binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. In a preferred embodiment, the binding domain lengths are at least 6 nucleotides. Preferably, both binding domains have a combined total length of at least 14 nucleotides. Various permutations in the length of the two binding domains, such as 7+7, 8+8 and 9+9, are envisioned. It is well established that the greater the binding domain length, the more tightly it will bind to its complementary mRNA sequence. Accordingly, in a more preferred embodiment, each domain is nine or more nucleotides in length.

Within the context of the present invention, preferred cleavage sites within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 are as follows:

(i) the GU site corresponding to nucleotides 198-199;
(ii) the GU site corresponding to nucleotides 200-201;
(iii) the GU site corresponding to nucleotides 264-265;
(iv) the AU site corresponding to nucleotides 271-272;
(v) the AU site corresponding to nucleotides 301-302;
(vi) the GU site corresponding to nucleotides 303-304; and
(vii) the AU site corresponding to nucleotides 316-317.

In a further preferred embodiment, the DNAzyme has a sequence selected from:

(i) 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO: 3)
targets GU (nt 198, 199); arms hybridise to bp 189-207

(ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO: 4)
targets GU (nt 200, 201); arms hybridise to bp 191-209

(iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO: 5)
targets GU (nt 264, 265); arms hybridise to bp 255-273

(iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6)
targets AU (nt 271, 272); arms hybridise to bp 262-280

(v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO: 7)
targets AU (nt 271, 272); arms hybridise to bp 262-280

(vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO: 8)
targets AU (nt 301, 302); arms hybridise to bp 292-310

(vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO: 9)
targets GU (nt 303, 304); arms hybridise to bp 294-312

(viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO: 10)
targets AU (nt 316, 317); arms hybridise to bp 307-325.

In a particularly preferred embodiment, the DNAzyme targets the AU site corresponding to nucleotides 271-272 (ie. the translation start codon).

In a further preferred embodiment, the DNAzyme has the sequence: 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6).

In applying DNAzyme-based treatments, it is preferable that the DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent phosphate bonding between the 3' carbons of the terminal nucleotide and its adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion". Accordingly, in a preferred embodiment, the 3'-end nucleotide residue is inverted in the binding domain contiguous with the 3' end of the catalytic domain. In addition to inversions, the instant DNAzymes may contain modified nucleotides. Modified nucleotides

include, for example, N3'-P5' phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art.

In a particularly preferred embodiment, the DNAzyme includes an inverted T at the 3' position.

As will be appreciated by those skilled in the art, given that DNAzymes of the present invention cleave human EGR-1, similar DNAzymes can be produced to cleave the corresponding mRNA in other species, eg. rat (NGFI-A), mouse (Egr-1) etc. In a further aspect, the present invention provides such DNAzymes.

In a second aspect the present invention provides a pharmaceutical composition including a DNAzyme according to the first aspect and a pharmaceutically acceptable carrier.

In a third aspect the present invention provides a method of inhibiting EGR-1 activity in cells which includes exposing the cells to a DNAzyme according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of inhibiting proliferation or migration of cells in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In a fifth aspect the present invention provides a method of treating a condition associated with cell proliferation or migration in a subject which includes administering to the subject a prophylactically effective dose of a DNAzyme according to the first aspect of the present invention.

In preferred embodiments of the third, fourth and fifth aspects of the present invention, the cells are vascular cells, particularly smooth muscle or endothelial cells. The cells may, however, be cells involved in neoplasia, such as tumour cells and the like.

Although the subject may be any animal or human, it is preferred that the subject is a human.

In a preferred embodiment, conditions associated with SMC proliferation(and migration) are selected from post-angioplasty restenosis, vein graft failure, transplant coronary disease and complications associated with atherosclerosis (cerebrovascular infarction (stroke), myocardial infarction (heart attack), hypertension or peripheral vascular disease (gangrene of the extremities).

Within the parameters of the fourth and fifth aspects of the present invention, any suitable mode of administration may be used to administer or deliver the DNAzyme.

In particular, delivery of the nucleic acid agents described may be achieved by one or more of the following methods:

(a) Liposomes and liposome-protein conjugates and mixtures.

(b) Using catheters to deliver intra-luminal formulations of the nucleic acid as a solution or in a complex with a liposome.

(c) Catheter delivery to adventitial tissue as a solution or in a complex with a liposome.

(d) Within a polymer formulation such as polyethylenimine (PEI) or pluronic gels or within ethylene vinyl acetate copolymer (EVAc). The polymer is preferably delivered intra-luminally.

(e) The nucleic acid may be bound to a delivery agent such as a targetting moiety, or any suitable carrier such as a peptide or fatty acid molecule.

(f) Within a viral-liposome complex, such as Sendai virus.

(g) The nucleic acid may be delivered by a double angioplasty balloon device fixed to catheter.

(h) The nucleic acid could be delivered on a specially prepared stent of the Schatz-Palmaz or derivative type. The stent could be coated with a polymer or agent impregnated with nucleic acid that allows controlled release of the molecules at the vessel wall.

In a preferred embodiment, the mode of administration is topical administration. Topical administration may be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The topical administration can be performed, for example, via catheter and topical injection, and via coated stent as discussed below.

Pharmaceutical carriers for topical administration are well known in the art, as are methods for combining same with active agents to be delivered. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition.

Topical delivery systems include, for example, gels and solutions, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic

polymers (e.g., polycarbophil and polyvinylpyrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a biodegradable polymer. Examples of agents which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,N^{I},N^{II},N^{III}-tetramethyl-N,N^{I},N^{II},N^{III}-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE) (GIBCO BRL); (2) Cytofection GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate) (Boehringer Mannheim); (4) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL); (5) FuGENE^{6} (Roche Molecular Biochemicals); (6) Superfect (Qiagen); and (7) Lipofectamine 2000 (Gibco-life Technologies).

Examples of suitable methods for topical administration of the DNAzymes of the present invention are described in Autieri et al. (1995), Simons et al. (1992), Morishita et al. (1993), Bennett and Schwartz (1995) and Frimerman et al. (1999).

Determining the prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective dose contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the prophylactically effective dose contains between about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective dose contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment, the prophylactically effective does contains about 25 mg of the instant DNAzyme.

In a sixth aspect the present invention provides an angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to the first aspect.

Angioplastic stents, also known by other terms such as "intravascular stents" or simple "stents", are well known in the art. They are routinely used to prevent vascular closure due to physical anomalies such as unwanted inward growth of vascular tissue due to surgical trauma. They often have a

tubular, expanding lattice-type structure appropriate for their function, and can optionally be biodegradable.

In this invention, the stent can be operably coated with the instant pharmaceutical composition using any suitable means known in the art. Here, "operably coating" a stent means coating it in a way that permits the timely release of the pharmaceutical composition into the surrounding tissue to be treated once the coated stent is administered. Such coating methods, for example, can use the polymer polypyrrole.

In a seventh aspect, the present invention provides a method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to the fifth aspect to the subject at around the time of the angioplasty.

As used herein, administration "at around the time of angioplasty" can be performed during the procedure, or immediately before or after the procedure. The administering can be performed according to known methods such as catheter delivery.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following non-limiting Figures and Examples.

Table 1

```
Symbol comparison table: GenRunData:pileupdna.cmp  CompCheck: 6876
                 GapWeight: 5.000
            GapLengthWeight: 0.300
EGR1align.msf  MSF: 4388  Type: N  April  7, 1998 12:07  Check: 5107

Name: mouseEGR1  Len:  4388  Check: 8340  Weight: 1.0(SEQ ID NO:11)
Name: ratEGR1    Len:  4388  Check: 8587  Weight: 1.0(SEQ ID NO:12)
Name: humanEGR1  Len:  4388  Check: 8180  Weight: 1.00 (SEQ ID NO:1)

NB. THIS IS RAT NGFI-A numbering
                1                                                    50
mouseEgr1       .......... .......... .......... .......... ..........
  ratNGFIA      CCGCGGAGCC TCAGCTCTAC GCGCCTGGCG CCCTCCCTAC GCGGGCGTCC
humanEGR1       .......... .......... .......... .......... ..........

               51                                                   100
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       CCGACTCCCG CGCGCGTTCA GGCTCCGGGT TGGGAACCAA GGAGGGGGAG
humanEGR1       .......... .......... .......... .......... ..........

              101                                                   150
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       GGTGGGTGCG CCGACCCGGA AACACCATAT AAGGAGCAGG AAGGATCCCC
humanEGR1       .......... .......... .......... .......... ..........

              151                                                   200
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       CGCCGGAACA GACCTTATTT GGGCAGCGCC TTATATGGAG TGGCCCAATA
humanEGR1       .......... .......... .......... .......... ..........

              201                                                   250
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       TGGCCCTGCC GCTTCCGGCT CTGGGAGGAG GGGCGAACGG GGGTTGGGGC
humanEGR1       .......... .......... .......... .......... ..........

              251                                                   300
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       GGGGGCAAGC TGGGAACTCC AGGAGCCTAG CCCGGGAGGC CACTGCCGCT
humanEGR1       .......... .......... .......... .......... ..........

              301                                                   350
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       GTTCCAATAC TAGGCTTTCC AGGAGCCTGA GCGCTCAGGG TGCCGGAGCC
humanEGR1       .......... .......... .......... .......... ..........

              351                                                   400
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       GGTCGCAGGG TGGAAGCGCC CACCGCTCTT GGATGGGAGG TCTTCACGTC
humanEGR1       .......... .......... .......... .......... ..........

              401                                                   450
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1       ACTCCGGGTC CTCCCGGTCG GTCCTTCCAT ATTAGGGCTT CCTGCTTCCC
humanEGR1       .......... .......... .......... .......... ..........

              451                                                   500
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mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  ATATATGGCC ATGTACGTCA CGGCGGAGGC GGGCCCGTGC TGTTTCAGAC
humanEGR1  .......... .......... .......... .......... ..........

           501                                                  550
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  CCTTGAAATA GAGGCCGATT CGGGGAGTCG CGAGAGATCC CAGCGCGCAG
humanEGR1  .......... .......... .......... .......... ....CCGCAG

           551                                                  600
mouseEGR1  .....GGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
  ratEGR1  AACTTGGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
humanEGR1  AACTTGGGGA GCCGCCGCCG CCATCCGCCG CCGCAGCCAG CTTCCGCCGC

           601                                                  650
mouseEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
  ratEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
humanEGR1  CGCAGGACCG GCCCCTGCCC CAGCCTCCGC AGCCGCGGCG CGTCCACGCC

           651                                                  700
mouseEGR1  GGGCCGCGGC TACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
  ratEGR1  GGGCCGCGGC CACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
humanEGR1  CGCCCGCGCC CAGGGCGAGT CGGGGTCGCC GCCTGCACGC TTCTCAGTGT

           701                                                  750
mouseEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CCCCCGGCGA GTGTGCCCTC
  ratEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CATCCGGCGA GTGTGCCCTC
humanEGR1  TCCCC.GCGC CCCGCATGTA ACCCGGCCAG GCCCCCGCAA CGGTGTCCCC

           751                                                  800
mouseEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGTTCTCCA
  ratEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGCTCTCCA
humanEGR1  TGCAGCTCCA GCCCCGGGCT GCACCCCCCC GCCCCGACAC CAGCTCTCCA

           801                                                  850
mouseEGR1  GCTCGCTGGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
  ratEGR1  GCTCGCACGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
humanEGR1  GCCTGCTCGT CCAGGATGGC CGCGGCCAAG GCCGAGATGC AGCTGATGTC

           ED5 (rat) arms hybridise to bp 807-825 in rat sequ
           hED5(hum) arms hybridise to bp 262-280 in hum sequ

           851                                                  900
mouseEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
  ratEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
humanEGR1  CCCGCTGCAG ATCTCTGACC CGTTCGGATC CTTTCCTCAC TCGCCCACCA

           901                                                  950
mouseEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
  ratEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
humanEGR1  TGGACAACTA CCCTAAGCTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT

           951                                                 1000
mouseEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGTAAT..
  ratEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGCAATAA
humanEGR1  CCCCAGTTCC TCGGCGCCGC CGGGGCCCCA GAGGGCAGCG GCAGCAACAG

           1001                                                1050
mouseEGR1  .......AGC AGCAGCAGCA CCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
  ratEGR1  CAGCAGCAGC AGCAGCAGCA GCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
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humanEGR1  CAGCAGCAGC AGCAGCGGGG GCGGTGGAGG CGGCGGGGGC GGCAGCAACA

           1051                                                1100
mouseEGR1  GCAACAGCGG CAGCAGCGCC TTCAATCCTC AAGGGGAGCC GAGCGAACAA
  ratEGR1  GCAACAGCGG CAGCAGCGCT TTCAATCCTC AAGGGGAGCC GAGCGAACAA
humanEGR1  GCAGCAGCAG CAGCAGCACC TTCAACCCTC AGGCGGACAC GGGCGAGCAG

           1101                                                1150
mouseEGR1  CCCTATGAGC ACCTGACCAC AG...AGTCC TTTTCTGACA TCGCTCTGAA
  ratEGR1  CCCTACGAGC ACCTGACCAC AGGTAAGCGG TGGTCTGCGC CGAGGCTGAA
humanEGR1  CCCTACGAGC ACCTGACCGC AG...AGTCT TTTCCTGACA TCTCTCTGAA

           1151                                                1200
mouseEGR1  TAATGAGAAG GCGATGGTGG AGACGAGTTA TCCCAGCCAA ACGACTCGGT
  ratEGR1  TCCCCCTTCG TGACTACCCT AACGTCCAGT CCTTTGCAGC ACGGACCTGC
humanEGR1  CAACGAGAAG GTGCTGGTGG AGACCAGTTA CCCCAGCCAA ACCACTCGAC

           1201                                                1250
mouseEGR1  TGCCTCCCAT CACCTATACT GGCCGCTTCT CCCTGGAGCC CGCACCCAAC
  ratEGR1  ATCTAGATCT TAGGGACGGG ATTGGGATTT CCCTCTATTC ..CACACAGC
humanEGR1  TGCCCCCCAT CACCTATACT GGCCGCTTTT CCCTGGAGCC TGCACCCAAC

           1251                                                1300
mouseEGR1  AGTGGCAACA CTTTGTGGCC TGAACCCCTT TTCAGCCTAG TCAGTGGCCT
  ratEGR1  TCCAGGGACT TGTGTTAGAG GGATGTCTGG GGACCCCCCA ACCCTCCATC
humanEGR1  AGTGGCAACA CCTTGTGGCC CGAGCCCCTC TTCAGCTTGG TCAGTGGCCT

           1301                                                1350
mouseEGR1  CGTGAGCATG ACCAATCCTC CGACCTCTTC ATCCTCGGCG CCTTCTCCAG
  ratEGR1  CTTGCGGGTG CGCGGAGGGC AGACCGTTTG TTTTGGATGG AGAACTCAAG
humanEGR1  AGTGAGCATG ACCAACCCAC CGGCCTCCTC GTCCTCAGCA CCATCTCCAG

           1351                                                1400
mouseEGR1  CTGCTTCATC GTCTTCCTCT GCCTCCCAGA GCCCGCCCCT GAGCTGTGCC
  ratEGR1  TTGCGTGGGT GGCT..... .....GGAGT GGGGGAGGGT TTGTTTTGAT
humanEGR1  CGGCCTCCTC CGC...CTCC GCCTCCCAGA GCCCACCCCT GAGCTGCGCA

           1401                                                1450
mouseEGR1  GTGCCGTCCA ACGACAGCAG TCCCATCTAC TCGGCTGCGC CCACCTTTCC
  ratEGR1  GAGCAGGGTT GC....CCCC TCCCCCGCGC GCGTTGTCGC GAGCCTTGTT
humanEGR1  GTGCCATCCA ACGACAGCAG TCCCATTTAC TCAGCGGCAC CCACCTTCCC

           1451                                                1500
mouseEGR1  TACTCCCAAC ACTGACATTT TTCCTGAGCC CCAAAGCCAG GCCTTTCCTG
  ratEGR1  TGCAGCTTGT TCCCAAGGAA GGGCTGAAAT CTGTCACCAG GGATGTCCCG
humanEGR1  CACGCCGAAC ACTGACATTT TCCCTGAGCC ACAAAGCCAG GCCTTCCCGG

           1501                                                1550
mouseEGR1  GCTCGGCAGG CACAGCCTTG CAGTACCCGC CTCCTGCCTA CCCTGCCACC
  ratEGR1  CCGCCCAGGG TAGGGGCGCG CATTAGCTGT GGCC.ACTAG GGTGCTGGCG
humanEGR1  GCTCGGCAGG GACAGCGCTC CAGTACCCGC CTCCTGCCTA CCCTGCCGCC

           1551                                                1600
mouseEGR1  AAAGGTGGTT TCCAGGTTCC CATGATCCCT GACTATCTGT TTCCACAACA
  ratEGR1  GGATTCCCTC ACCCCGGACG CCTGCTGCGG AGCGCTCTCA GAGCTGCAGT
humanEGR1  AAGGGTGGCT TCCAGGTTCC CATGATCCCC GACTACCTGT TTCCACAGCA

           1601                                                1650
mouseEGR1  ACAGGGAGAC CTGAGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGTC
  ratEGR1  AGAGGGGGAT TCTCTGTTTG CGTCAGCTGT CGAAATGGCT CT......GC
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humanEGR1  GCAGGGGGAT CTGGGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGCC

           1651                                                1700
mouseEGR1  TGGAGAACCG TACCCAGCAG CCTTCGCTCA CTCCACTATC CACTATTAAA
  ratEGR1  CACTGGAGCA GGTCCAGGAA CATTGCAATC TGCTGCTATC AATTATTAAC
humanEGR1  TGGAGAGCCG CACCCAGCAG CCTTCGCTAA CCCCTCTGTC TACTATTAAG

           1701                                                1750
mouseEGR1  GCCTTCGCCA CTCAGTCGGG CTCCCAGGAC TTAAAG.... ...GCTCTTA
  ratEGR1  CACATCGAGA GTCAGTGGTA GCCGGGCGAC CTCTTGCCTG GCCGCTTCGG
humanEGR1  GCCTTTGCCA CTCAGTCGGG CTCCCAGGAC CTGAAG.... ...GCCCTCA

           1751                                                1800
mouseEGR1  ATACCACCTA CCAATCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT
  ratEGR1  CTCTCATCGT CCAGTGATTG CTCTCCAGTA ACCAGGCCTC TCTGTTCTCT
humanEGR1  ATACCAGCTA CCAGTCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT

           1801                                                1850
mouseEGR1  ACCCCAACCG GCCCAGCAAG ACACCCCCCC ATGAACGCCC ATATGCTTGC
  ratEGR1  TTCCTGCCAG AGTCCTTTTC TGACATCGCT CTGAATAACG AGAAG..GCG
humanEGR1  ATCCCAACCG GCCCAGCAAG ACGCCCCCCC ACGAACGCCC TTACGCTTGC

           1851                                                1900
mouseEGR1  CCTGTCGAGT CCTGCGATCG CCGCTTTTCT CGCTCGGATG AGCTTACCCG
  ratEGR1  CTGGTGGAGA CAAGTTATCC CAGCCAAACT ACCCGGTTGC CTCCCATCAC
humanEGR1  CCAGTGGAGT CCTGTGATCG CCGCTTCTCC CGCTCCGACG AGCTCACCCG

           1901                                                1950
mouseEGR1  CCATATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGT CGAATCTGCA
  ratEGR1  CTATACTGGC CGCTTCTCCC TGGAGCCTGC ACCCAACAGT GGCAACACTT
humanEGR1  CCACATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGC CGCATCTGCA

           1951                                                2000
mouseEGR1  TGCGTAACTT CAGTCGTAGT GACCACCTTA CCACCCACAT CCGCACCCAC
  ratEGR1  TGTGGCCTGA ACCCCTTTTC AGCCTAGTCA GTGGCCTTGT GAGCATGACC
humanEGR1  TGCGCAACTT CAGCCGCAGC GACCACCTCA CCACCCACAT CCGCACCCAC

           2001                                                2050
mouseEGR1  ACAGGCGAGA AGCCTTTTGC CTGTGACATT TGTGGGAGGA AGTTTGCCAG
  ratEGR1  AACCCTCCAA CCTCTTCATC CTCAGCGCCT TCTCCAGCTG CTTCATCGTC
humanEGR1  ACAGGCGAAA AGCCCTTCGC CTGCGACATC TGTGGAAGAA AGTTTGCCAG

           2051                                                2100
mouseEGR1  GAGTGATGAA CGCAAGAGGC ATACCAAAAT CCATTTAAGA CAGAAGGACA
  ratEGR1  TTCCTCTGCC TCCCAGAGCC CACCCCTGAG CTGTGCCGTG CCGTCCAACG
humanEGR1  GAGCGATGAA CGCAAGAGGC ATACCAAGAT CCACTTGCGG CAGAAGGACA

           2101                                                2150
mouseEGR1  AGAAAGCAGA CAAAAGTGTG GTGGCCTCCC CGGCTGC... .CTCTTCACT
  ratEGR1  ACAGCAGTCC CATTTACTCA GCTGCACCCA CCTTTCCTAC TCCCAACACT
humanEGR1  AGAAAGCAGA CAAAAGTGTT GTGGCCTCTT CGGCCACCTC CTCTCTCTCT

           2151                                                2200
mouseEGR1  .......... .......... CTCTTCTTAC CCATCCCCAG TGGCTACCTC
  ratEGR1  .......... .......... GACATTTTTC CTGAGCCCCA AAGCCAGGCC
humanEGR1  TCCTACCCGT CCCCGGTTGC TACCTCTTAC CCGTCCCCGG TTACTACCTC

           2201                                                2250
mouseEGR1  CTACCCATCC CCTGCCACCA CCTCATTCCC ATCCCCTGTG CCCACTTCCT
  ratEGR1  TTTCCTGGCT CTGCAGGCAC AGCCTTGCAG TACCCGCCTC CTGCCTACCC
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          humanEGR1  TTATCCATCC CCGGCCACCA CCTCATACCC ATCCCCTGTG CCCACCTCCT

                     2251                                                2300
          mouseEGR1  ACTCCTCTCC TGGCTCCTCC ACCTACCCAT CTCCTGCGCA CAGTGGCTTC
5           ratEGR1  TGCCACCAAG GGTGGTTTCC AGGTTCCCAT GATCCCTGAC TATCTGTTTC
          humanEGR1  TCTCCTCTCC CGGCTCCTCG ACCTACCCAT CCCCTGTGCA CAGTGGCTTC

                     2301                                                2350
          mouseEGR1  CCGTCGCCGT CAGTGGCCAC CACCTTTGCC TCCGTTCC.. ..........
10          ratEGR1  CACAACAACA GGGAGACCTG AGCCTGGGCA CCCCAGACCA GAAGCCCTTC
          humanEGR1  CCCTCCCCGT CGGTGGCCAC CACGTACTCC TCTGTTCCC. ..........

                     2351                                                2400
          mouseEGR1  ....ACCTGC TTTCCCCACC CAGGTCAGCA GCTTCCCGTC TGCGGGCGTC
15          ratEGR1  CAGGGTCTGG AGAACCGTAC CCAGCAGCCT TCGCTCACTC CACTATCCAC
          humanEGR1  .....CCTGC TTTCCCGGCC CAGGTCAGCA GCTTCCCTTC CTCAGCTGTC

                     2401                                                2450
          mouseEGR1  AGCAGCTCCT TCAGCACCTC AACTGGTCTT TCAGACATGA CAGCGACCTT
20          ratEGR1  TATCAAAGCC TTCGCCACTC AGTCGGGCTC CCAGGACTTA AAGGCTCTTA
          humanEGR1  ACCAACTCCT TCAGCGCCTC CACAGGGCTT TCGGACATGA CAGCAACCTT

                     2451                                                2500
          mouseEGR1  TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGA....... .ATAAAAG..
25          ratEGR1  ATAACACCTA CCAGTCCCAA CTCATCAAAC CCAGCCGCAT GCGCAAGT..
          humanEGR1  TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGAAAGGGGA AAGAAAGGGA

                     2501                                                2550
          mouseEGR1  .AAAGCAAAG GGAGAGGCAG GAAAGACATA AAAGCA...C AGGAGGGAAG
30          ratEGR1  .ACCCCAACC GGCCCAGCAA GACACCCCCC CATGAACGCC CGTATGCTTG
          humanEGR1  AAAGGGAGAA AAAGAAACAC AAGAGACTTA AAGGACAGGA GGAGGAGATG

                     2551                                                2600
          mouseEGR1  AGATGGCCGC AAGAGGGGCC ACCTCTTAGG TCAGATGGAA GATCTCAGAG
35          ratEGR1  CCCTGTTGAG TCCTGCGATC GCCGCTTTTC TCGCTCGGAT GAGCTTACAC
          humanEGR1  GCCATAGGAG AGGAGGGTT. .CCTCTTAGG TCAGATGGAG GTTCTCAGAG

                     2601                                                2650
          mouseEGR1  CCAAGTCCTT CTACTCACGA GTA..GAAGG ACCGTTGGCC AACAGCCCTT
40          ratEGR1  GCCACATCCG CATCCATACA GGC..CAGAA GCCCTTCCAG TGTCGAATCT
          humanEGR1  CCAAGTCCTC CCTCTCTACT GGAGTGGAAG GTCTATTGGC CAACAATCCT

                     2651                                                2700
          mouseEGR1  TCACTTACCA TCCCTGCCTC CCCCGTCCTG TTCCCTTTGA CTTCAGCTGC
45          ratEGR1  GCATGCGTAA TTTCAGTCGT AGTGACCACC TTACCACCCA CATCCGCACC
          humanEGR1  TTCTGCCCAC TTCCCCTTCC CCAATTACTA TTCCCTTTGA CTTCAGCTGC

                     2701                                                2750
          mouseEGR1  CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG GACTTGATTT
50          ratEGR1  C..ACACAGG CGAGAAGCCT TTTGCCTGTG ACATTTGTGG GAGAAAGTTT
          humanEGR1  CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG AACTTGATTT

                     2751                                                2800
          mouseEGR1  GCATGG.... ..TATTGGAT AAATCATTTC AGTATCCTCT ..........
55          ratEGR1  GCCAGGAGTG ATGAACGCAA GAGGCATACC AAAATCCACT TAAGACAGAA
          humanEGR1  GCATGGA... ..TTTTGGAT AAATCATTTC AGTATCATCT ..........

                     2801                                                2850
          mouseEGR1  .....CCATC ACATGCCTGG CCCTTGCTCC CTTCAGCGCT AGACCATCAA
60          ratEGR1  GGACAAGAAA GCAGACAAAA GTGTCGTGGC CTCCTCAGCT GCCTCTTCCC
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humanEGR1  ....CCATCA TATGCCTGAC CCCTTGCTCC CTTCAATGCT AGAAAATCGA

           2851                                                2900
mouseEGR1  GTTGGCATAA AGAAAAAAAA ATGGGTTTGG GCCCTCAGAA CCCTGCCCTG
  ratEGR1  TCTCTTCCTA CCCATCCCCA GTGGCTACCT CCTACCCATC CCCCGCCACC
humanEGR1  GTTGGC.... .....AAAAT GGGGTTTGGG CCCCTCAGAG CCCTGCCCTG

           2901                                                2950
mouseEGR1  CATCTTTGTA CAGCATCTGT GCCATGGATT TTGTTTTCCT TGGGGTATTC
  ratEGR1  ACCTCATTTC CATCCCCAGT GCCCACCTCT TACTCCTCTC CGGGCTCCTC
humanEGR1  CACCCTTGTA CAGTGTCTGT GCCATGGATT TCGTTTTTCT TGGGGTACTC

           2951                                                3000
mouseEGR1  TTGATGTGAA GATAATTTGC ATACT..... .CTATTGTAT TATTTGGAGT
  ratEGR1  TACCTACCCG TCTCCTGCAC ACAGTGGCTT CCCATCGCCC TCGGTGGCCA
humanEGR1  TTGATGTGAA GATAATTTGC ATATT..... .CTATTGTAT TATTTGGAGT

           3001                                                3050
mouseEGR1  TAAATCCTCA CTTTGGGG.. GAGGGGGGAG CAAAGCCAAG CAAACCAATG
  ratEGR1  CCACCTATGC CTCCGTCC.. CACCTGCTTT CCCTGCCCAG GTCAGCACCT
humanEGR1  TAGGTCCTCA CTTGGGGGAA AAAAAAAAAA AAAAGCCAAG CAAACCAATG

           3051                                                3100
mouseEGR1  ATGATCCTCT ATTTTGTGAT GACTCTGCTG TGACATTA.. ..........
  ratEGR1  TCCAGTCTGC AGGGGTCAGC AACTCCTTCA GCACCTCAAC GGGTCTTTCA
humanEGR1  GTGATCCTCT ATTTTGTGAT GATGCTGTGA CAATA..... ..........

           3101                                                3150
mouseEGR1  .GGTTTGAAG CATTTTTTTT TTCAAGCAGC AGTCCTAGGT ATTAACTGGA
  ratEGR1  GACATGACAG CAACCTTTTC TCCTAGGACA ATTGAAATTT GCTAAAGGGA
humanEGR1  ...AGTTTGA ACCTTTTTTT TTGAAACAGC AGTCCCAG.. ..TATTCTCA

           3151                                                3200
mouseEGR1  ..GCATGTGT CAGAGTGTTG TTCCGTTAAT TTTGTAAATA CTGGCTCGAC
  ratEGR1  ATGAAAGAGA GCAAAGGGAG GGGAGCGCGA GAGACAATAA AGGACAGGAG
humanEGR1  GAGCATGTGT CAGAGTGTTG TTCCGTTAAC CTTTTTGTAA ATACTGCTTG

           3201                                                3250
mouseEGR1  .TGTAACTCT CACATGTGAC AAAGTATGGT TTGTTTGGTT GGGTTTTGTT
  ratEGR1  .GGAAGAAAT GGCCCGCAAG AGGGGCTGCC TCTTAGGTCA GATGGAAGAT
humanEGR1  ACCGTACTCT CACATGTGGC AAAATATGGT TTGGTTTTTC TTTTTTTTTT

           3251                                                3300
mouseEGR1  TTTGAGAATT TTTTTGCCCG TCCCTTTGGT TTCAAAAGTT TCACGTCTTG
  ratEGR1  CTCAGAGCCA AGTCCTTCTA GTCAGTAGAA GGCCCGTTGG CCACCAGCCC
humanEGR1  TTGAAAGTGT TTTTTCTTCG TCCTTTTGGT TTAAAAAGTT TCACGTCTTG

           3301                                                3350
mouseEGR1  GTGCCTTTTG TGTGACACGC CTT.CCGATG GCTTGACATG CGCA......
  ratEGR1  TTTCACTTAG CGTCCCTGCC CTC.CCCAGT CCCGGTCCTT TTGACTTCAG
humanEGR1  GTGCCTTTTG TGTGATGCCC CTTGCTGATG GCTTGACATG TGCAAT....

           3351                                                3400
mouseEGR1  ...GATGTGA GGGACACGCT CACCTTAGCC TTAA...GGG GGTAGGAGTG
  ratEGR1  CTGCCTGAAA CAGCCACGTC CAAGTTCTTC ACCT...CTA TCCAAAGGAC
humanEGR1  .....TGTGA GGGACATGCT CACCTCTAGC CTTAAGGGGG GCAGGGAGTG

           3401                                                3450
mouseEGR1  ATGTGTTGGG GGAGGCTTGA GAGCAAAAAC GAGGAAGAGG GCTGAGCTGA
  ratEGR1  TTGATTTGCA TGGTATTGGA TAAACCATTT CAGCATCATC TCCACCACAT
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humanEGR1  ATGATTTGGG GGAGGCTTTG GGAGCAAAAT AAGGAAGAGG GCTGAGCTGA

           3451                                                 3500
mouseEGR1  GCTTTCGGTC TCCAGAATGT AAGAAGAAAA AATTTAAACA AAAATCTGAA
ratEGR1    GCCTGGCCCT TGCTCCCTTC AGCACTAGAA CATCAAGTTG GCTGAAAAAA
humanEGR1  GCTTCGGTTC TCCAGAATGT AAGAAAACAA AATCTAAAAC AAAATCTGAA

           3501                                                 3550
mouseEGR1  CTCTCAAAAG TCTATTTTTC TAAACTGAAA ATGTAAATTT ATACATCTAT
ratEGR1    AAAATGGGTC TGGGCCCTCA GAACCCTGCC CTGTATCTTT GTACA.....
humanEGR1  CTCTCAAAAG TCTATTTTTT TAA.CTGAAA ATGTAAATTT ATAAATATAT

           3551                                                 3600
mouseEGR1  TCAGGAGTTG GAGTGTTGTG GTTACCTACT GAGTAGGCTG CAGTTTTTGT
ratEGR1    GCATCTGTGC CATGGATTTT GTTTTCCTTG GGGTATTCTT GATGTGAAGA
humanEGR1  TCAGGAGTTG GAATGTTGTA GTTACCTACT GAGTAGGCGG CGATTTTTGT

           3601                                                 3650
mouseEGR1  ATGTTATGAA CATGAAGTTC ATTATTTTGT GGTTTTATTT TACTTTGTAC
ratEGR1    TAATTTGCAT ACTCTATTGT ACTATTTGGA GTTAAATTCT CACTTTGGGG
humanEGR1  ATGTTATGAA CATGCAGTTC ATTATTTTGT GGTTCTATTT TACTTTGTAC

           3651                                                 3700
mouseEGR1  TTGTGTTTGC TTAAACAAAG TAACCTGTTT GGCTTATAAA CACATTGAAT
ratEGR1    GAGGGGGAGC AAAGCCAAGC AAACCAATGG TGATCCTCTA TTTTGTGATG
humanEGR1  TTGTGTTTGC TTAAACAAAG TGA.CTGTTT GGCTTATAAA CACATTGAAT

           3701                                                 3750
mouseEGR1  GCGCTCTATT GCCCATGG.. ..GATATGTG GTGTGTATCC TTCAGAAAAA
ratEGR1    ATCCTGCTGT GACATTAGGT TTGAAACTTT TTTTTTTTTT TGAAGCAGCA
humanEGR1  GCGCTTTATT GCCCATGG.. ..GATATGTG GTGTATATCC TTCCAAAAAA

           3751                                                 3800
mouseEGR1  TTAAAAGGAA AAAT..... ..........  .......... ..........
ratEGR1    GTCCTAGGTA TTAACTGGAG CATGTGTCAG AGTGTTGTTC CGTTAATTTT
humanEGR1  TTAAAACGAA AATAAAGTAG CTGCGATTGG G.........  ..........

           3801                                                 3850
mouseEGR1  ..........  ..........  ..........  ..........  ..........
ratEGR1    GTAAATACTG CTCGACTGTA ACTCTCACAT GTGACAAAAT ACGGTTTGTT
humanEGR1  ..........  ..........  ..........  ..........  ..........

           3851                                                 3900
mouseEGR1  ..........  ..........  ..........  ..........  ..........
ratEGR1    TGGTTGGGTT TTTTGTTGTT TTTGAAAAAA AAATTTTTTT TTTGCCCGTC
humanEGR1  ..........  ..........  ..........  ..........  ..........

           3901                                                 3950
mouseEGR1  ..........  ..........  ..........  ..........  ..........
ratEGR1    CCTTTGGTTT CAAAAGTTTC ACGTCTTGGT GCCTTTGTGT GACACACCTT
humanEGR1  ..........  ..........  ..........  ..........  ..........

           3951                                                 4000
mouseEGR1  ..........  ..........  ..........  ..........  ..........
ratEGR1    GCCGATGGCT GGACATGTGC AATCGTGAGG GGACACGCTC ACCTCTAGCC
humanEGR1  ..........  ..........  ..........  ..........  ..........

           4001                                                 4050
mouseEGR1  ..........  ..........  ..........  ..........  ..........
ratEGR1    TTAAGGGGGT AGGAGTGATG TTTCAGGGGA GGCTTTAGAG CACGATGAGG
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```
humanEGR1  .......... .......... .......... .......... ..........

           4051                                                4100
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  AAGAGGGCTG AGCTGAGCTT TGGTTCTCCA GAATGTAAGA AGAAAAATTT
humanEGR1  .......... .......... .......... .......... ..........

           4101                                                4150
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  AAAACAAAAA TCTGAACTCT CAAAAGTCTA TTTTTTTAAC TGAAAATGTA
humanEGR1  .......... .......... .......... .......... ..........

           4151                                                4200
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  GATTTATCCA TGTTCGGGAG TTGGAATGCT GCGGTTACCT ACTGAGTAGG
humanEGR1  .......... .......... .......... .......... ..........

           4201                                                4250
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  CGGTGACTTT TGTATGCTAT GAACATGAAG TTCATTATTT TGTGGTTTTA
humanEGR1  .......... .......... .......... .......... ..........

           4251                                                4300
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  TTTTACTTCG TACTTGTGTT TGCTTAAACA AAGTGACTTG TTTGGCTTAT
humanEGR1  .......... .......... .......... .......... ..........

           4301                                                4350
mouseEGR1  .......... .......... .......... .......... ..........
  ratEGR1  AAACACATTG AATGCGCTTT ACTGCCCATG GGATATGTGG TGTGTATCCT
humanEGR1  .......... .......... .......... .......... ..........

           4351                                    4388
mouseEGR1  .......... .......... .......... ........
  ratEGR1  TCAGAAAAAT TAAAAGGAAA ATAAAGAAAC TAACTGGT
humanEGR1  .......... .......... .......... ........
```

Example 1

Characterisation of DNAzymes ED5 and hED5

Materials and Methods

ODN synthesis. DNAzymes were synthesized commercially (Oligos Etc., Inc.) with an inverted T at the 3' position unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated ODNs were 5'-end labeled with γ^{32}P-dATP and T4 polynucleotide kinase (New England Biolabs). Unincorporated label was separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech).

In vitro transcript and cleavage experiments. A ^{32}P-labelled 206 nt NGFI-A RNA transcript was prepared by in vitro transcription (T3 polymerase) of plasmid construct pJDM8 (as described in Milbrandt, 1987, the entire contents of which are incorporated herein by reference) previously cut with *Bgl* II. Reactions were performed in a total volume of 20 μl containing 10 mM $MgCl_2$, 5 mM Tris pH 7.5, 150 mM NaCl, 4.8 pmol of in vitro transcribed or synthetic RNA substrate and 60 pmol DNAzyme (1:12.5 substrate to DNAzyme ratio), unless otherwise indicated. Reactions were allowed to proceed at 37 °C for the times indicated and quenched by transferring an aliquot to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

Culture conditions and DNAzyme transfection. Primary rat aortic SMCs were obtained from Cell Applications, Inc., and grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50 μg/ml streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. SMCs were used in experiments between passages 3-7. Pup rat SMCs (WKY12-22 (as described in Lemire et al, 1994, the entire contents of which are incorporated herein by reference)) were grown under similar conditions. Subconfluent (60-70%) SMCs were incubated in serum-free medium (SFM) for 6 h prior to DNAzyme (or antisense ODN, where indicated) transfection (0.1 μM) using Superfect in accordance with manufacturer's instructions (Qiagen). After 18 h, the cells were washed with phosphate-buffered saline (PBS), pH 7.4 prior to transfection a second time in 5% FBS.

Northern blot analysis. Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25 μg was resolved by electrophoresis prior to transfer to Hybond-N+ membranes (NEN-DuPont). Prehybridization, hybridization with α^{32}P-dCTP-labeled Egr-1 or β-Actin cDNA, and washing was performed essentially as previously described (Khachigian et al, 1995).

Western blot analysis. Growth-quiescent SMCs in 100 mm plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μg/ml leupeptin, 1% aprotinin and 2 mM PMSF. Twenty four μg protein samples were loaded onto 10% denaturing SDS-polyacrylamide gels and electroblotted onto PVDF nylon membranes (NEN-DuPont). Membranes were air dried prior to blocking with non-fat skim milk powder in PBS containing 0.05% (w:v) Tween 20. Membranes were incubated with rabbit antibodies to Egr-1 or Sp1 (Santa Cruz Biotechnology, Inc.) (1:1000) then with HRP-linked mouse anti-rabbit Ig secondary antiserum (1:2000). Where mouse monoclonal c-Fos (Santa Cruz Biotechnology, Inc.) was used, detection was achieved with HRP-linked rabbit anti-mouse Ig. Proteins were visualized by chemiluminescent detection (NEN-DuPont).

Assays of cell proliferation. Growth-quiescent SMCs in 96-well titer plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, trypsinized and the suspension was quantitated using an automated Coulter counter.

Assessment of DNAzyme stability. DNAzymes were 5'-end labeled with γ^{32}P-dATP and separated from free label by centrifugation. Radiolabeled DNAzymes were incubated in 5% FBS or serum-free medium at 37 °C for the times indicated. Aliquots of the reaction were quenched by transfer to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were applied to 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

SMC wounding assay. Confluent growth-quiescent SMCs in chamber slides (Nunc-InterMed) were exposed to ED5 or ED5SCR for 18 h prior to a single scrape with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20 μM) for 2 h prior to injury (Pitsch et al, 1996; Horodyski &

Powell, 1996). Seventy-two h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde then stained with hematoxylin-eosin.

Rat arterial ligation model and analysis. Adult male Sprague Dawley rats weighing 300-350 g were anaesthetised using ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right common carotid artery was exposed up to the carotid bifurcation via a midline neck incision. Size 6/0 non-absorbable suture was tied around the common carotid proximal to the bifurcation, ensuring cessation of blood flow distally. A 200 μl solution at 4°C containing 500 μg of DNAzyme (in DEPC-treated H_2O), 30 μl of transfecting agent and Pluronic gel P127 (BASF) was applied around the vessel in each group of 5 rats, extending proximally from the ligature for 12-15 mm. These agents did not inhibit the solidification of the gel at 37 °C. After 3 days, vehicle with or without 500 μg of DNAzyme was administered a second time. Animals were sacrificed 18 days after ligation by lethal injection of phenobarbitone, and perfusion fixed using 10% (v:v) formaldehyde perfused at 120 mm Hg. Both carotids were then dissected free and placed in 10% formaldehyde, cut in 2 mm lengths and embedded in 3% (w:v) agarose prior to fixation in paraffin. Five μm sections were prepared at 250 μm intervals along the vessel from the point of ligation and stained with hematoxylin and eosin. The neointimal and medial areas of 5 consecutive sections per rat were determined digitally using a customized software package (Magellan) (Halasz & Martin, 1984) and expressed as a mean ratio per group of 5 rats.

Results and Discussion

The 7x7 nt arms flanking the 15 nt DNAzyme catalytic domain in the original DNAzyme design 7 were extended by 2 nts per arm for improved specificity (L.-Q. Sun, data not shown) (Figure 1). The 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (T) to confer resistance to 3'->5' exonuclease digestion. The sequence in both arms of ED5 was scrambled (SCR) without altering the catalytic domain to produce DNAzyme ED5SCR (Figure 1).

A synthetic RNA substrate comprised of 23 nts, matching nts 805 to 827 of NGFI-A mRNA (Figure 1) was used to determine whether ED5 had the capacity to cleave target RNA. ED5 cleaved the ^{32}P-5'-end labeled 23-mer within 10 min. The 12-mer product corresponds to the length between the

A(816)-U(817) junction and the 5' end of the substrate (Figure 1). In contrast, ED5SCR had no demonstrable effect on this synthetic substrate. Specific ED5 catalysis was further demonstrated by the inability of the human equivalent of this DNAzyme (hED5) to cleave the rat substrate over a wide range of stoichiometric ratios. Similar results were obtained using ED5SCR (data not shown). hED5 differs from the rat ED5 sequence by 3 of 18 nts in its hybridizing arms (Table 2). The catalytic effect of ED5 on a ^{32}P-labeled 206 nt fragment of native NGFI-A mRNA prepared by *in vitro* transcription was then determined. The cleavage reaction produced two radiolabeled species of 163 and 43 nt length consistent with DNAzyme cleavage at the A(816)-U(817) junction. In other experiments, ED5 also cleaved a ^{32}P-labeled NGFI-A transcript of 1960 nt length in a specific and time-dependent manner (data not shown).

Table 2. DNAzyme target sites in mRNA.

Similarity between the 18 nt arms of ED5 or hED5 and the mRNA of rat NGFI-A or human EGR-1 (among other transcription factors) is expressed as a percentage. The target sequence of ED5 in NGFI-A mRNA is 5'-807-A CGU CCG GGA UGG CAG CGG-825-3' (SEQ ID NO: 13) (rat NGFI-A sequence), and that of hED5 in EGR-1 is 5'-262-U CGU CCA GGA UGG CCG CGG-280-3' (SEQ ID NO: 14) (Human EGR-1 sequence). Nucleotides in bold indicate mismatches between rat and human sequences. Data obtained by a gap best fit search in ANGIS using sequences derived from Genbank and EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

Gene	Accession number	Best homology over 18 nts (%)	
		ED5	hED5
Rat NGFI-A	M18416	**100**	84.2
Human EGR-1	X52541	84.2	**100**
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9

To determine the effect of the DNAzymes on endogenous levels of NGFI-A mRNA, growth-quiescent SMCs were exposed to ED5 prior to stimulation with serum. Northern blot and densitometric analysis revealed that ED5 (0.1 μM) inhibited serum-inducible steady-state NGFI-A mRNA levels by 55% (Figure 2a), whereas ED5SCR had no effect (Figure 2a). The capacity of ED5 to inhibit NGFI-A synthesis at the level of protein was assessed by Western blot analysis. Serum-induction of NGFI-A protein was suppressed by ED5. In contrast, neither ED5SCR nor EDC, a DNAzyme bearing an identical catalytic domain as ED5 and ED5SCR but flanked by nonsense arms had any influence on the induction of NGFI-A (data not

shown). ED5 failed to affect levels of the constitutively expressed, structurally -related zinc-finger protein, Sp1. It was also unable to block serum-induction of the immediate-early gene product, c-Fos whose induction, like NGFI-A, is dependent upon serum response elements in its promoter and phosphorylation mediated by extracellular-signal regulated kinase (Treisman, 1990, 1994 and 1995; Gashler & Sukhatme, 1995). These findings, taken together, demonstrate the capacity of ED5 to inhibit production of NGFI-A mRNA and protein in a gene-specific and sequence-specific manner, consistent with the lack of significant homology between its target site in NGFI-A mRNA and other mRNA (Table 2).

The effect of ED5 on SMC replication was then determined. Growth-quiescent SMCs were incubated with DNAzyme prior to exposure to serum and the assessment of cell numbers after 3 days. ED5 (0.1 μM) inhibited SMC proliferation stimulated by serum by 70% (Figure 3a). In contrast, ED5SCR failed to influence SMC growth (Figure 3a). AS2, an antisense NGFI-A ODN able to inhibit SMC growth at 1 μM failed to inhibit proliferation at the lower concentration (Figure 3a). Additional experiments revealed that ED5 also blocked serum-inducible ^{3}H-thymidine incorporation into DNA (data not shown). ED5 inhibition was not a consequence of cell death since no change in morphology was observed, and the proportion of cells incorporating Trypan Blue in the presence of serum was not influenced by either DNAzyme (Figure 3b).

Cultured SMCs derived from the aortae of 2 week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries (Seifert et al, 1984; Majesky et al, 1992). The epitheloid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media (Majesky et al, 1988). WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules (Lemire et al, 1994), such as NGFI-A (Rafty & Khachigian, 1998), consistent with a "synthetic" phenotype (Majesky et al, 1992; Campbell & Campbell, 1985). ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 75% (Figure 3c). ED5SCR had no inhibitory effect; surprisingly, it appeared to stimulate growth (Figure 3c). Trypan Blue exclusion revealed that DNAzyme inhibition was not a consequence of cytotoxicity (data not shown).

To ensure that differences in the biological effects of ED5 and ED5SCR were not the consequence of dissimilar intracellular localization, both DNAzymes were 5'-end labeled with fluorescein isothiocyanate (FITC) and incubated with SMCs. Fluorescence microscopy revealed that both FITC-ED5 and FITC-ED5SCR localized mainly within the nuclei. Punctate fluorescence in this cellular compartment was independent of DNAzyme sequence. Fluorescence was also observed in the cytoplasm, albeit with less intensity. Cultures not exposed to DNAzyme showed no evidence of autofluorescence.

Both molecules were 5'-end labeled with γ^{32}P-dATP and incubated in culture medium to ascertain whether cellular responsiveness to ED5 and ED5SCR was a consequence of differences in DNAzyme stability. Both ^{32}P-ED5 and ^{32}P-ED5SCR remained intact even after 48 h. In contrast to ^{32}P-ED5 bearing the 3' inverted T, degradation of ^{32}P-ED5 bearing its 3' T in the correct orientation was observed as early as 1 h. Exposure to serum-free medium did not result in degradation of the molecule even after 48 h. These findings indicate that inverse orientation of the 3' base in the DNAzyme protects the molecule from nucleolytic cleavage by components in serum.

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to either DNAzyme and Mitomycin C, an inhibitor of proliferation (Pitsch et al, 1996; Horodyski & Powell, 1996) prior to scraping. Cultures in which DNAzyme was absent repopulated the entire denuded zone within 3 days. ED5 inhibited this reparative response to injury and prevented additional growth in this area even after 6 days (data not shown). That ED5SCR had no effect in this system further demonstrates sequence-specific inhibition by ED5.

The effect of ED5 on neointima formation was investigated in a rat model. Complete ligation of the right common carotid artery proximal to the bifurcation results in migration of SMCs from the media to the intima where proliferation eventually leads to the formation of a neointima (Kumar & Lindner, 1997; Bhawan et al, 1977; Buck, 1961). Intimal thickening 18 days after ligation was inhibited 50% by ED5 (Figure 4). In contrast, neither its scrambled counterpart (Figure 4) nor the vehicle control (Figure 4) had any effect on neointima formation. These findings demonstrate the capacity of

ED5 to suppress SMC accumulation in the vascular lumen in a specific manner, and argue against inhibition as a mere consequence of a "mass effect" (Kitze et al, 1998; Tharlow et al, 1996).

Further experiments revealed the capacity of hED5 to cleave (human) EGR-1 RNA. hED5 cleaved its substrate in a dose-dependent manner over a wide range of stoichiometric ratios. hED5 also cleaved in a time-dependent manner, whereas hED5SCR, its scrambled counterpart, had no such catalytic property (data not shown).

The specific, growth-inhibitory properties of ED5 reported herein suggest that DNAzymes may be useful as therapeutic tools in the treatment of vascular disorders involving inappropriate SMC growth.

Example 2

Cleavage of human EGR-1 RNA by panel of candidate DNAzymes

To evaluate which specific DNAzymes targeting human EGR-1 (other than hED5) efficiently cleave EGR-1 RNA, we prepared *in vitro* transcribed 35S-labeled EGR-1 RNA and incubated this substrate with candidate DNAzymes for various times. The EGR-1 plasmid template (hs164) was prepared by subcloning bps 168-332 of human EGR-1 into pGEM-T-easy. A 388 nt 35S-labeled substrate was prepared by *in vitro* transcription using SP6 polymerase. Time-dependent cleavage of the substrate was tested using the following DNZzymes:

DzA: 5'-CAGGGGACAGGCTAGCTACAACGACGTTGCGGG-X-3' (SEQ ID NO: 15) ;

DzB: 5'-TGCAGGGGAGGCTAGCTACAACGAACCGTTGCG-X-3' (SEQ ID NO: 16) ;

DzC: 5'-CATCCTGGAGGCTAGCTACAACGAGAGCAGGCT-X-3' (SEQ ID NO: 17) ;

DzE: 5'-TCAGCTGCAGGCTAGCTACAACGACTCGGCCTT-X-3' (SEQ ID NO: 18) ; and

DzF: 5'-GCGGGGACAGGCTAGCTACAACGACAGCTGCAT-X-3' (SEQ ID NO: 19)

where X denotes a 3'-3-linked T.

The DNAzyme that cleaved most effectively of this group was DzA, then DzB, then DzC. In contrast, DzE was inactive.

Example 3

Inhibition of induction of EGR-1 in human SMC by DzA

To determine whether DzA could block the induction of endogenous human EGR-1, we incubated growth-quiescent human aortic smooth muscle cells with 5% fetal bovine serum and observed the production of EGR-1 protein by Western blot analysis. This band representing the EGR-1 protein was blocked by 0.5 μM DzA, delivered using FuGENE6 (Roche Molecular Biochemicals) and unaffected by DzE. The blot was then stripped and reprobed with antibodies to the transcription factor Spl. Results obtained showed that neither serum nor DzA affected induction of Sp1. A Coomassie Blue gel indicated that equal protein had been loaded.

The data demonstrate that DzA cleaves EGR-1 mRNA and blocks the induction of EGR-1 protein.

Example 4

Inhibition of human SMC proliferation by DzA

To ascertain whether proliferation of human SMCs could be inhibited by DzA, a population of SMCs was quantitated with and without exposure to DzA or DzE. SMC proliferation stimulated by 5% fetal bovine serum was significantly inhibited by 0.5 μM DzA (Figure 5). In contrast, neither DzE nor ED5SCR had any effect (Figure 5). These data demonstrate that DzA inhibits human SMC proliferation.

Example 5

Inhibition of porcine SMC proliferation by DzA

The porcine and human EGR-1 sequences are remarkably well conserved (91%). Porcine retinal SMCs were used to determine whether DzA could block the growth of porcine SMCs. Our studies indicate that DzA (0.5 μM) could inhibit the proliferation of these cells (Figure 6). In contrast, DzE had no effect (Figure 6).

Example 6

Delivery of DNAzyme into the porcine coronary artery wall

Porcine angioplasty and stenting are accepted models of human in-stent restenosis (Karas et al. 1992). The porcine coronary anatomy, dimensions and histological response to stenting are similar to the human (Muller et al. 1992). The Transport Catheter has previously been used to deliver antisense DNA targeting c-*myc* in humans (Serrys et al. 1998) and the pig (Gunn & Cumberland, 1996) via the intraluminal route. Using this catheter, FITC-labeled DNAzyme was applied to the inner wall of a porcine coronary artery, *ex vivo*, from a newly explanted pig heart. DNAzyme (1000 µg) was delivered via the catheter in 2ml MilliQ H20 containing 300µl FuGENE6 and 1mM $MgC1_2$. The FITC-labeled DNAzyme localised into the intimal cells of the vessel wall. These studies demonstrate that DNAzyme can be delivered to cells within the artery wall via an intraluminal catheter.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive. In addition, various documents are cited throughout this application. The disclosures of these documents are hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

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Wilcox, J.N., Smith, K.M., Williams, L.T., Schwartz, S.M. & Gordon, D. (1988) Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. J. Clin. Invest. 82, 1134-1143.

Claims:

1. A DNAzyme which specifically cleaves EGR-1 mRNA, the DNAzyme comprising

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to the two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR-1 mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:1, such that the DNAzyme cleaves the EGR-1 mRNA.

2. A DNAzyme as claimed in claim 1 wherein each binding domain is nine or more nucleotides in length.

3. A DNAzyme as claimed in claim 1 or claim 2 in which the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO: 2).

4. A DNAzyme as claimed in any one of claims 1 to 3 in which the cleavage site is selected from the group consisting of

(i) the GU site corresponding to nucleotides 198-199;
(ii) the GU site corresponding to nucleotides 200-201;
(iii) the GU site corresponding to nucleotides 264-265;
(iv) the AU site corresponding to nucleotides 271-272;
(v) the AU site corresponding to nucleotides 301-302;
(vi) the GU site corresponding to nucleotides 303-304; and
(vii) the AU site corresponding to nucleotides 316-317.

5. A DNAzyme as claimed in claim 4 in which the cleavage site is the AU site corresponding to nucleotides 271-272.

6. A DNAzyme as claimed in claim 1 which has a sequence selected from the group consisting of:

(i) 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO: 3);
(ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO: 4);
(iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO: 5);
(iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6);
(v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO: 7);
(vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO: 8);
(vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO: 9); and
(viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO: 10).

7. A DNAzyme as claimed in claim 6 which has the sequence: 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO: 6).

8. A DNAzyme as claimed in any one of claims 1 to 7, wherein the 3'-end nucleotide residue is inverted in the binding domain contiguous with the 3' end of the catalytic domain.

9. A pharmaceutical composition comprising a DNAzyme according to any one of claims 1 to 8 and a pharmaceutically acceptable carrier.

10. A method of inhibiting EGR-1 activity in cells which comprises exposing the cells to a DNAzyme according to any one of claims 1 to 8.

11. A method of inhibiting proliferation or migration of cells in a subject which comprises administering to the subject a prophylactically effective dose of the pharmaceutical composition according to claim 9.

12. A method of treating a condition associated with cell proliferation or migration in a subject which comprises administering to the subject a therapeutically effective dose of the pharmaceutical composition according to claim 9.

13. A method as claimed in any one of claims 10 to 12 wherein the cells are vascular cells.

14. A method as claimed in any one of claims 10 to 12 wherein the cells are cells involved in neoplasia.

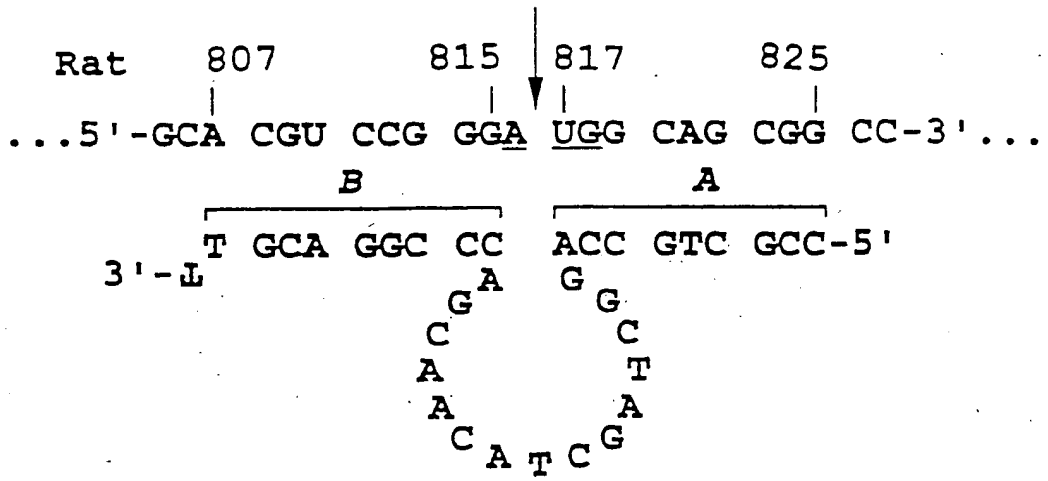
15. A method as claimed in claim 12 wherein the condition associated with cell proliferation or migration is selected from the group consisting of post-angioplasty restenosis, vein graft failure, hypertension, transplant coronary disease and complications associated with atherosclerosis or peripheral vascular disease.

16. An angioplastic stent for inhibiting the onset of restenosis, which comprises an angioplastic stent operably coated with a prophylactically effective dose of a DNAzyme according to any one of claims 1 to 8.

17. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a prophylactically effective dose of a pharmaceutical composition according to claim 9 to the subject at around the time of the angioplasty.

18. A method according to claim 17 in which the pharmaceutical composition is administered by catheter.

19. A method for inhibiting the onset of restenosis in a subject undergoing angioplasty, which comprises topically administering a stent according to claim 15 to the subject at around the time of the angioplasty.



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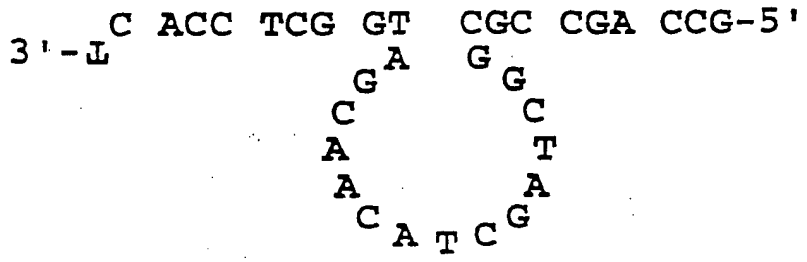

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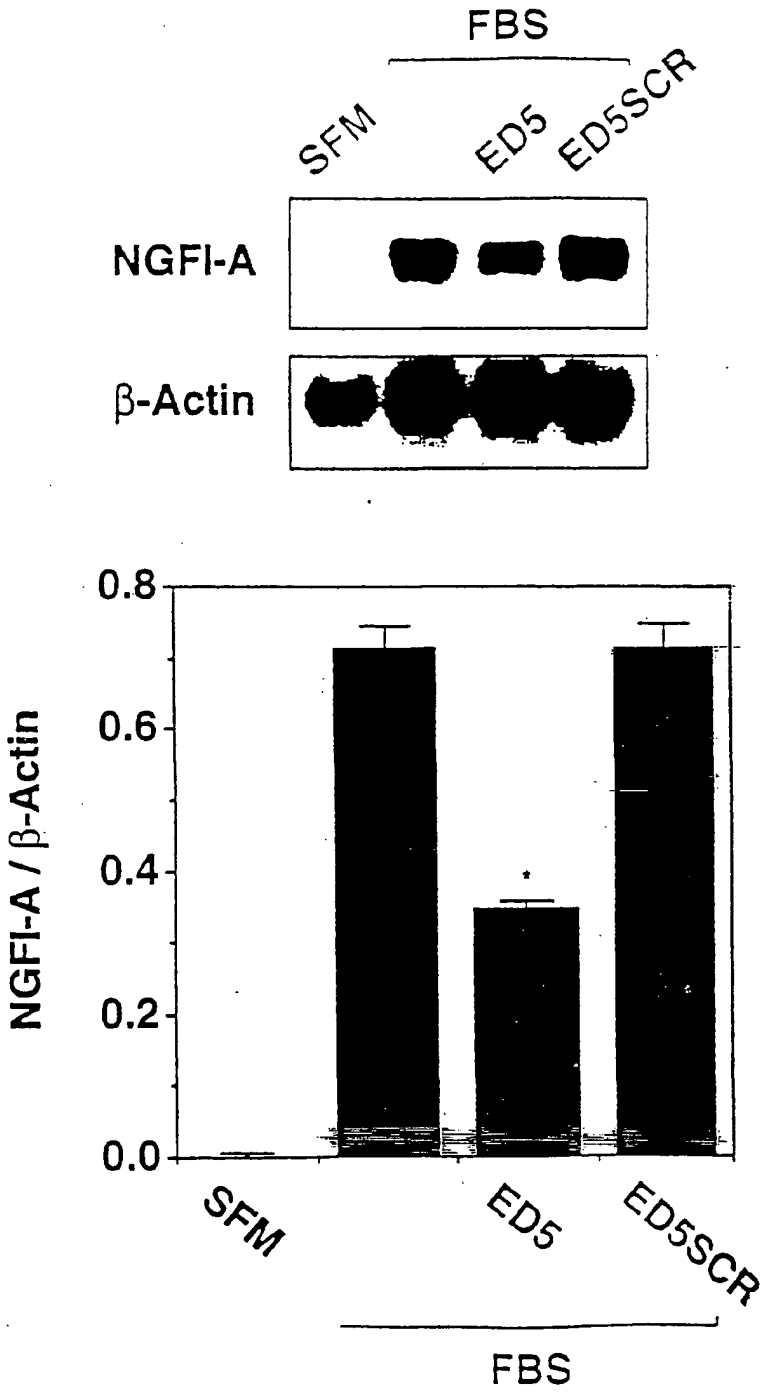
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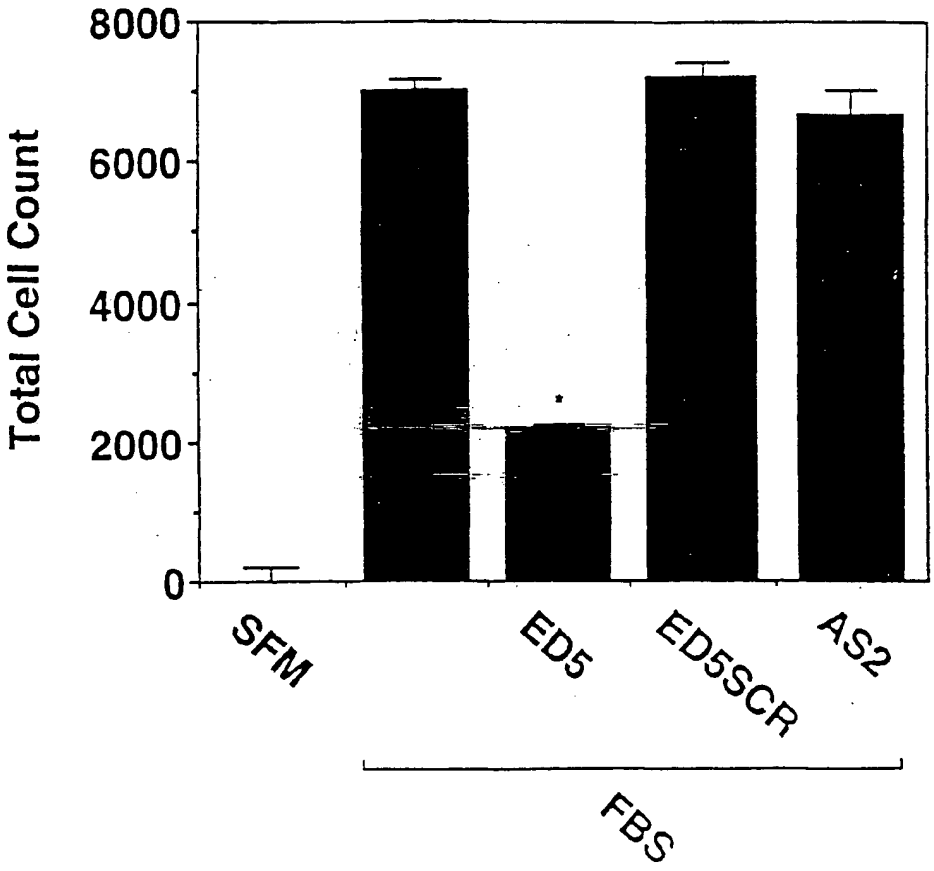

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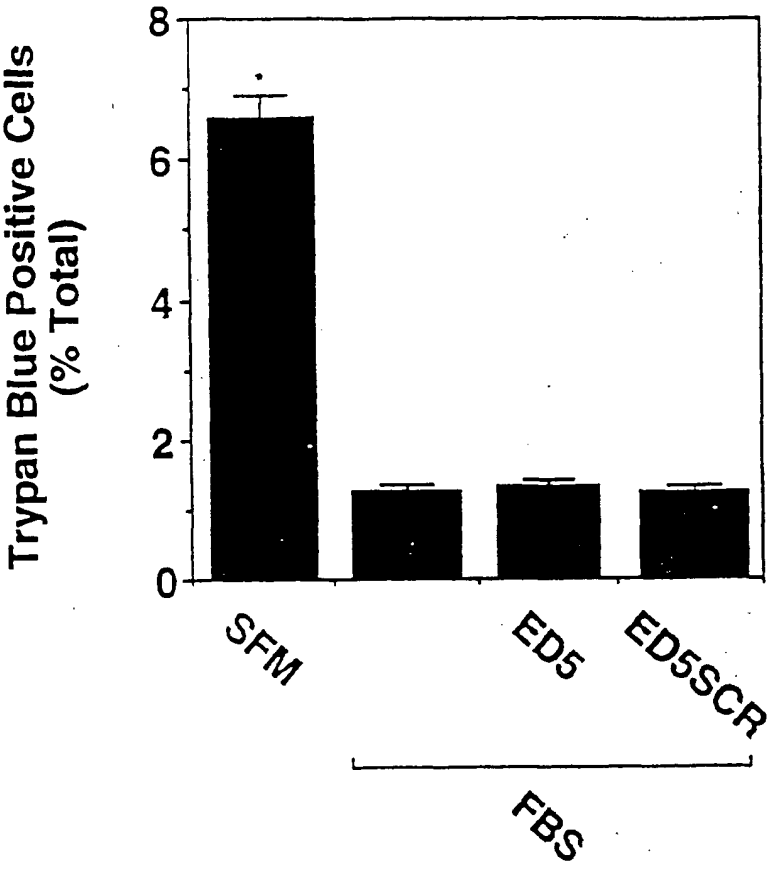

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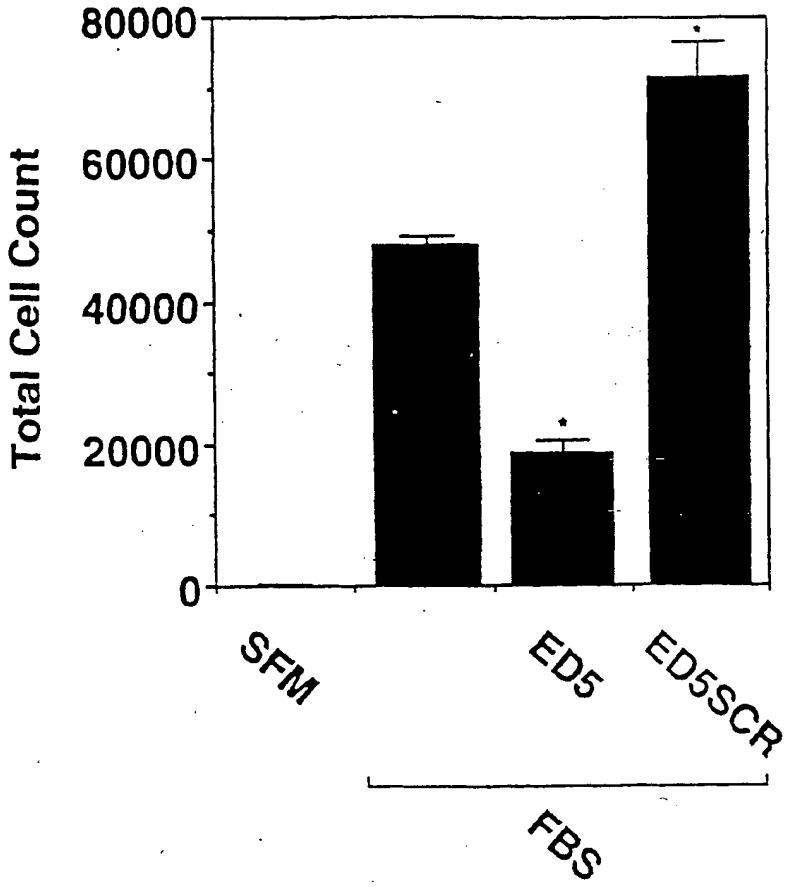
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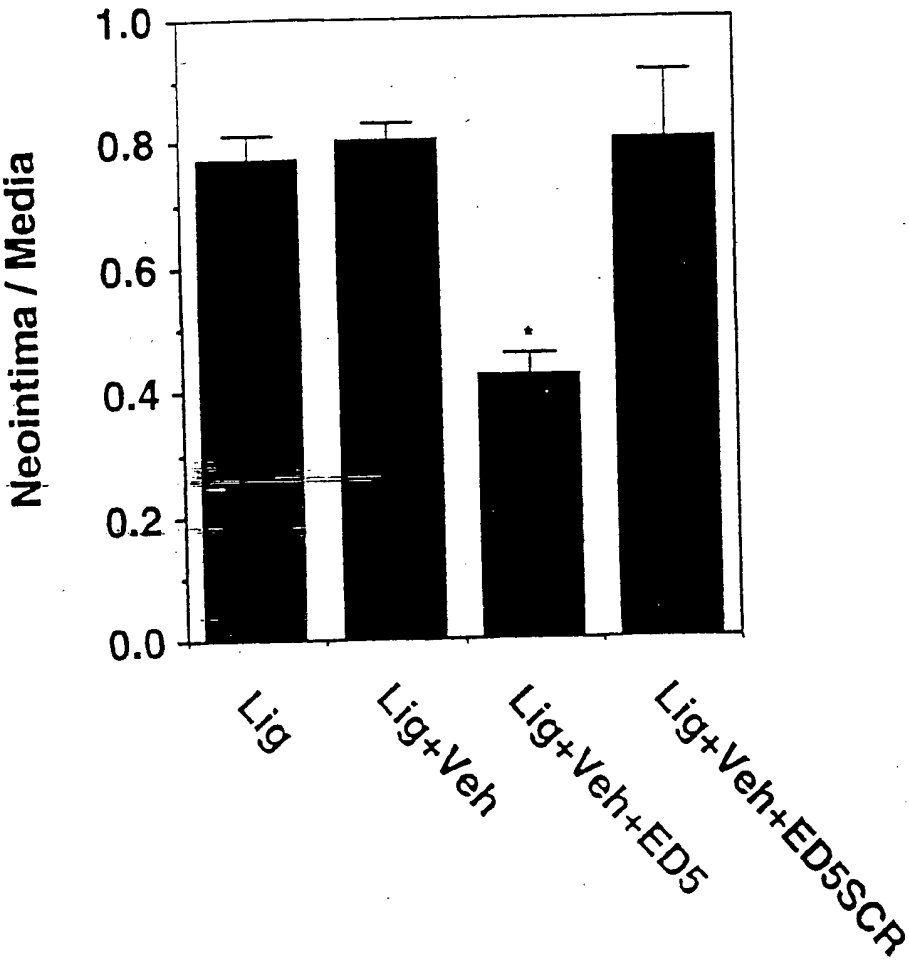
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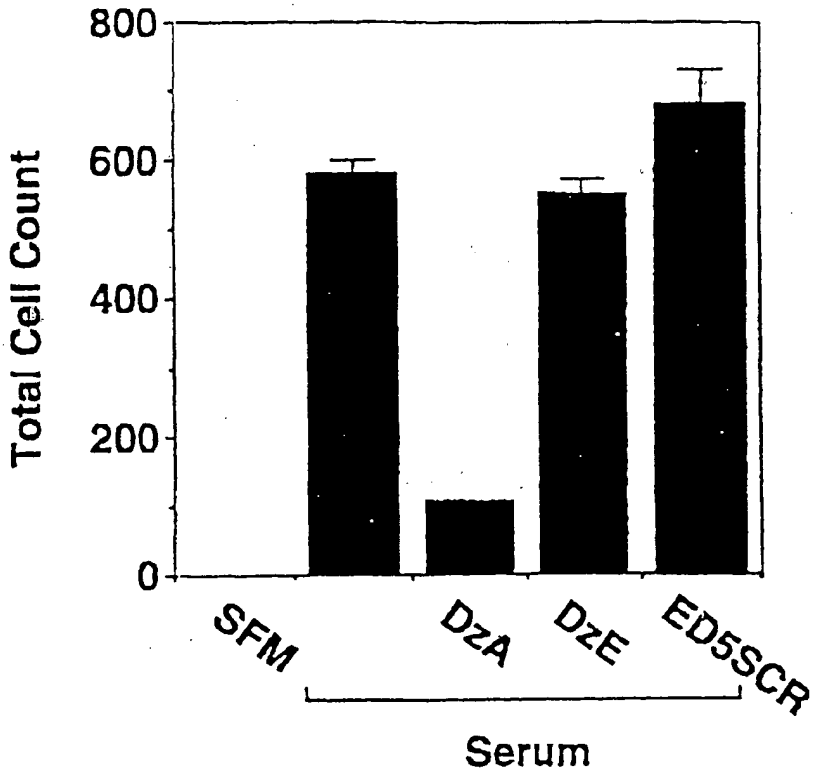
Figure 4



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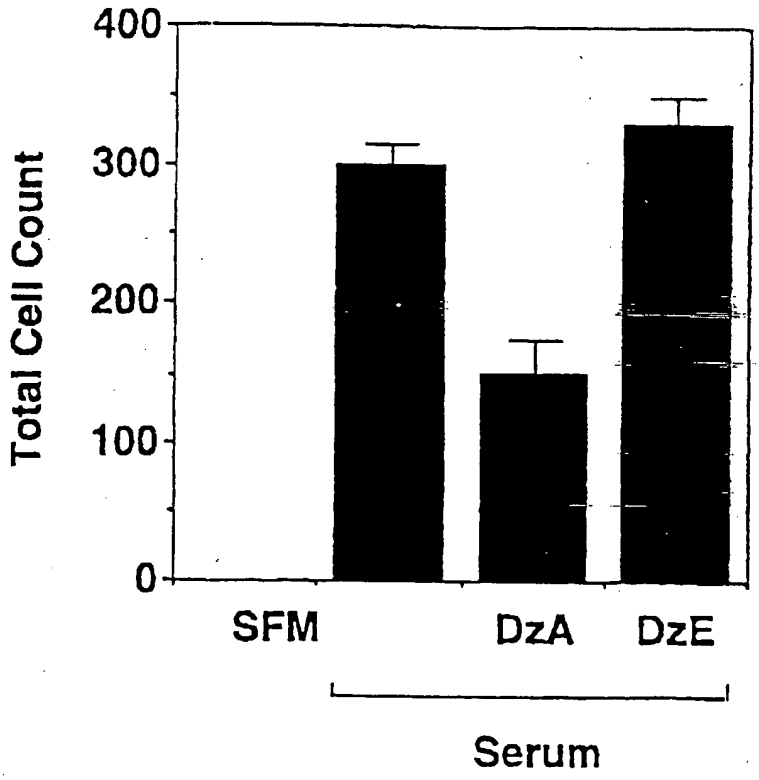

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00011

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 9/16; A61K 38/46; A61l 27/34, 33/12; C12Q 1/68.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
World Patent Index and Chemical Abstracts. Keywords: See electronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Medline, Biological Abstracts and Biotechnology Abstracts. Keywords: See electronic database box below.
EMBL, Genebank, DDBJ, PDB and Dgene(Derwent sequence database)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(DNAzyme# or deoxyribozyme# or DNA()enzyme# or catalytic(10n)DNA) and (EGR()1 or early()growth()response# or ngf? or zif268 or tis8 or krox24 or cef5 or cet5). Nucleic acid Sequence SEQ ID NO:2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Santiago F S *et al* "New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury." Nature Medicine volume 5 number 11 November 1999 pages 1264-1269. See the whole article especially figure 1.	1-15
X,Y	WO 97/32979 (UNISEARCH LIMITED) 12 September 1997. See whole document especially pages 4-6 and claims.	1-15
PY	Cairns M. J. *et al* "Target site selection for an RNA-cleaving catalytic DNA" Nature Biotechnology vol 17, May 1999 pages 480-486. See the whole document. Category Y with document D2.	1-8

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 March 2000	- 9 MAR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer J.H. CHAN Telephone No.: (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00011

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 98/49346 (THE SCRIPPS RESEARCH INSTITUTE) 5 November 1998.. See the whole document, especially pages 95-98, sequence id no. 85 and figures 8-10. Category Y with document D2.	1-8. 9-15.
X, Y	WO 96/17086 (THE SCRIPPS RESEARCH INSTITUTE) 6 June 1996. See the whole document, especially pages 11, 12, 32, 51-54, sequence id no. 85 and figures 8 and 9. Category Y with document D2.	1-8. 9-15.
X, Y	Santoro S W and Joyce G F "A general purpose RNA-cleaving DNA enzyme" Proc Natl Acad Sci USA volume 94 pages 4262-4266 April 1997. See the whole document, especially pp 4264-6 and figure 2. Category Y with document D2.	1-8. 9-15.
X, Y	Santoro S W and Joyce G F "Mechanism and utility of an RNA-cleaving DNA enzyme" Biochemistry 1998 September 22, 37, 13330-42 See the whole document especially pages 13331, 13337-41 and figure 1. Category Y with document D2.	1-8. 9-15
P, Y	WO 99/50452 (JOHNSON & JOHNSON RESEARCH PTY LIMITED) 7 October 1999. See the whole document, especially pages 17-25. Category Y with document D2.	1-8.
Y	Genebank accession no. X52541 Publication date 12 September 1993. Category Y with document D2.	1-8.
Y	Genebank accession no. X52541 Publication date 12 September 1993. Category Y with document D2.	1-8.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/00011

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	97/32979	AU	20865/97	CA	2248350	EP	934404
WO	98/49346	AU	72675/98	EP	981646		
WO	96/17086	AU	45950/96	BR	9510003	CA	2205382
		CN	1173207	EP	792375	FI	972333
		HU	77576	NO	972483	US	5807718
WO	99/50452	AU	35303/99				

END OF ANNEX